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Exploring and Exploiting the Protein S100A7 as a New Target for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR:  
Nathan West, B.Sc

CONTRACTING ORGANIZATION:  
British Columbia Cancer Agency  
Vancouver, BC, V5Z 1L3

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14. ABSTRACT S100A7 is strongly correlated with loss of estrogen receptor alpha (ER) expression in vivo. ER is absent in ~30% of breast cancers, is correlated with poor prognosis, and is a vital marker for response to endocrine therapy. Despite its importance, little is known about the mechanisms that drive the ER- phenotype. Our previous work demonstrated that S100A7 expression can originate from inflammatory stress, and we hypothesized that this could also regulate ER. We have found that the inflammatory cytokine oncostatin-M (OSM) can potently suppress ER expression in MCF7 and T47D breast cancer cells in vitro, while this stimulus strongly enhances S100A7 expression. ER suppression originates at the mRNA level in a mechanism dependent on canonical MAP kinase signaling through the MEKK-ERK1/2 pathway. Loss of ER inhibits the ability of cells to proliferate and express key ER-target genes such as progesterone receptor in response to estrogen. In addition to ER suppression and S100A7 induction, OSM induces changes characteristic of the epithelial to mesenchymal transition (EMT), a process associated with metastasis in vivo. These include loss of E-cadherin and gain of key mesenchymal transcription factors such as snail and twist. Importantly, high expression of the oncostatin-M receptor (OSMR) in patient samples is strongly associated with poor prognosis and downregulation of ER activity, in accordance with our experimental observations.					
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## Introduction

Breast cancer is a highly heterogeneous disease with respect to both clinical biomarkers and molecular expression patterns. There are currently at least five subtypes of breast cancer that are reproducibly identifiable by gene expression profiling, including the Luminal A, Luminal B, Her2+, basal-like, and normal-breast-like subtypes [1]. A key biomarker relevant to both clinical and molecular breast cancer classification is estrogen receptor alpha (ER). ER is expressed in ~70% of breast cancers, where it is correlated with relatively favorable prognosis and sensitivity to endocrine therapy. Its absence, a key feature of basal-like and many Her2+ tumors, is correlated with aggressive behaviour, resistance to endocrine therapy, and poor outcome [2, 3]. Despite the importance of ER, the biological processes regulating its expression (and the development of associated phenotypes) are not well characterized [2]. Understanding the events that shape the evolution of distinct breast cancer phenotypes is a central problem in breast cancer biology, the elucidation of which could have profound implications for treatment of the disease [4]. Findings described in our last annual report (and published in *Oncogene*, 2010; vol 29, 2083-92; see Appendix A of 2009 annual report) demonstrated that oncostatin-M (OSM), an inflammatory cytokine of the IL-6 family, can robustly induce expression of S100A7, a pro-survival gene in breast cancer that is correlated closely with ER negativity [5-7]. Because inflammation is a common feature of ER- tumors, we posed the hypothesis that OSM, in addition to regulating S100A7, may play an important role in driving acquisition of an ER- phenotype. The work described herein pertains to this question.

## Body—progress report pertaining to months 12 to 24 in the revised SOW

Significant progress was made on the aims outlined in Part A of the revised SOW (proposed in January 2010) during the last reporting period (months 1-12), a primary deliverable being the publication referenced above. Experimental work during the current reporting period was focused primarily on completing aims described in ***Part B—S100A7, inflammation, and ER regulation***, and will be described in detail herein.

For reference, the revised statement of work is provided on the following page:

## Statement of Work—Revised 2010

Months	Tasks
	<b>Part A. Extracellular roles of S100A7 in the breast tumor microenvironment.</b>
1-4	1. IHC staining and analysis of breast tumor TMA to identify infiltrating leukocyte subsets which correlate with S100A7 expression (if quality of existing TMA is poor, more time will be required (~2 months) to build and assemble a new cohort of cases for TMA, from tissues banked at the Manitoba and BCCA tumor tissue repositories);
5-16	2. Investigation of chemotactic responses of leukocyte subsets identified in TMA to soluble S100A7 using transwell and transendothelial migration assays;
5-24	3. Treatment of breast cell lines with cytokines (those known to be produced by leukocytes identified in TMA) to explore effects of leukocytes on S100A7 expression, as well as the relevant signal transduction pathways affecting S100A7;
12-24	4. Investigation of activation/effector responses of leukocytes identified in TMA to soluble S100A7 using flow cytometry (to detect markers of activation following treatment of human peripheral blood leukocytes with S100A7);
24-36	5. Based on data from the above tasks, planning and execution of further experiments to delve deeper into the extracellular roles of S100A7 in the tumor microenvironment.
	<b>Part B. S100A7, inflammation, and ER regulation.</b>
12-24	1. Elucidation of signaling/regulatory mechanisms controlling S100A7, ER, and EMT gene expression downstream of inflammatory cytokine signaling. Also, delineation of the functional relevance of S100A7 in this process.
12-36	2. Determination of cellular origins of OSM/OSMR in breast cancer.
12-36	3. Determination of the association of OSM/OSMR/S100A7 with molecular subtypes of breast cancer.
12-36	4. Determination of the significance of OSM/OSMR/S100A7 in relation to endocrine therapy.
	<u>Outcomes and deliverables:</u> S100A7 is known to affect immune activity in skin and is associated with inflammation in breast cancer. This study will address the potential immunomodulatory role of S100A7 in the breast tumor microenvironment. Specifically, this study will determine the following: (i) which leukocyte subtypes are specifically associated with S100A7 expression in the breast; (ii) the underlying mechanism of this association (i.e., recruitment of leukocytes due to S100A7 expression, S100A7 expression due to signals from leukocytes, or both), and (iii) the biological responses of specific leukocyte subtypes to soluble S100A7. In addition, we will examine the biological and clinical relevance of S100A7 in the context of ER regulation and EMT-like phenotypes downstream of inflammatory cytokine signaling. Furthermore, as this field is currently unexplored, this study will provide a basis for a potentially broad range of future investigations, and will support the potential exploitation of S100A7 as a target for breast cancer therapy.

### **Task A1**

Completed—see 2009 annual report, pages 5-6. Data are ready for publication pending generation of sufficient levels of accompanying mechanistic data.

### **Task A2**

Not yet initiated. While the recombinant S100A7 produced by our collaborators was very useful in our original structural studies (see 2009 report, pages 4-5), it was of bacterial origin. Because mammalian cells (particularly leukocytes) are extremely sensitive to LPS, we were not comfortable using this reagent in the context of assays involving live human cells. Furthermore, we have observed that S100A7 derived from human cell lysates frequently migrates to molecular weights in polyacrylamide gels that are higher than expected based on its amino acid sequence. This raises the possibility that S100A7 is post-transcriptionally modified by human cells, which may play an important role in its extracellular activities. As such, to avoid generation of misleading results, we have reluctantly postponed execution of this aim until we have a reliable source of human-derived S100A7. Currently, members of our lab have optimized a human protein expression system based on HEK293 cells (human embryonic kidney cells) and have successfully expressed several human proteins in this system. Efforts are now underway to produce HEK-derived S100A7 for use in our proposed aims involving S100A7 stimulation of breast tumor cells and leukocytes.

### **Task A3**

Completed and described largely in the 2009 annual report (Appendix A). Results from this aim were published in the following manuscript:

West, NR and Watson, PH. 2010. S100A7 (psoriasin) is induced by the proinflammatory cytokines oncostatin-M and interleukin-6 in human breast cancer. *Oncogene* **29**; 2083-92.

### **Task A4/A5**

Not yet initiated. For explanation, see discussion of Task A2.

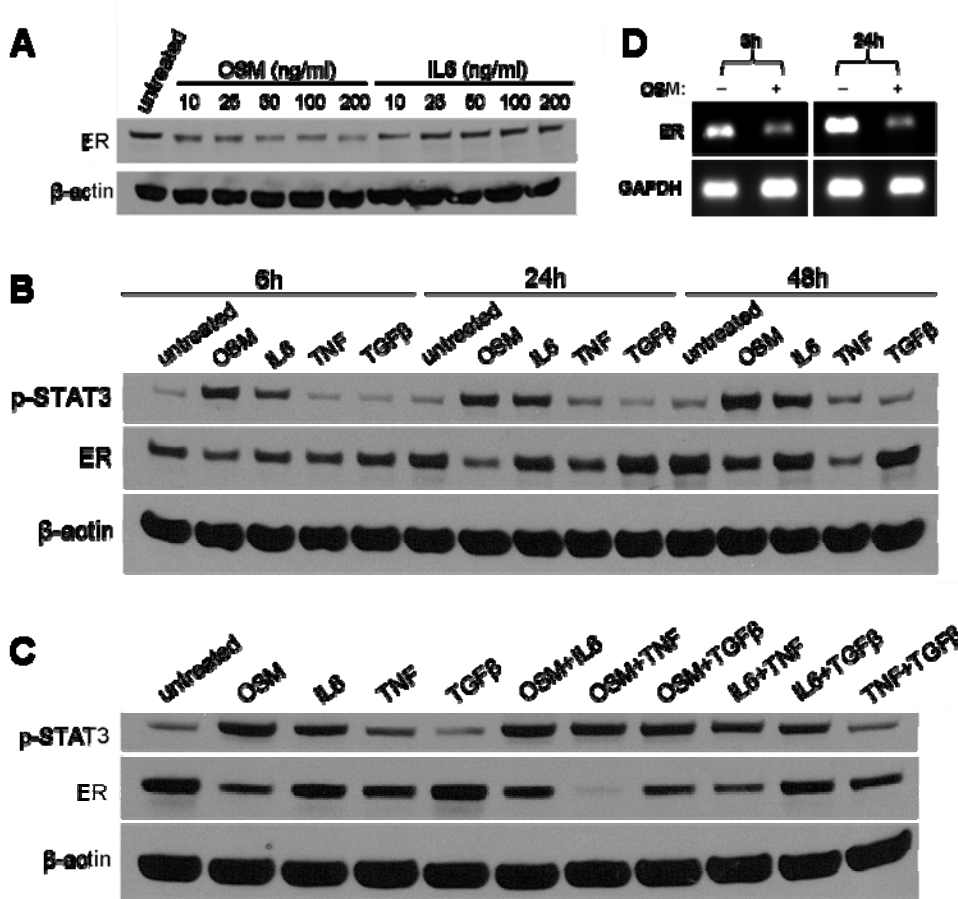
### **Task B1**

#### *OSM induces dose and time-dependent loss of ER*

The primary aim of this task was to determine whether inflammatory cytokines (with a focus on OSM) can regulate the expression of ER. To begin to address this we treated MCF7 and T47D cells with escalating doses of OSM or IL6 for 48h and observed a dose-dependent suppression of ER expression by western blot with OSM treatment, but no clear effect from IL6 stimulation (Fig. 1a). Maximal suppression of ER by OSM was attained at concentrations of 100ng/ml (this concentration was used for all subsequent experiments). We went on to treat MCF7 cells with 100ng/ml of OSM, IL-6, TNF, or TGF $\beta$  for 6, 24, or 48h, to compare the effects of OSM with those of other cytokines. OSM was the most potent suppressor of ER at early time points (6 and 24h), while TNF exerted suppression at 48h. IL6 and TGF $\beta$  had negligible effects at all time points (Fig. 1b). When cytokines were administered in combination, OSM and TNF synergistically suppressed ER and upregulated S100A7 (as we have reported previously; Fig. 1c). Although the effects of OSM and TNF on ER were very pronounced, we did not pursue this observation further, as prolonged incubation with both OSM and TNF significantly diminished cell viability. Suppression of ER expression occurred at the mRNA level as early as 3h post-stimulation, as assessed by RT-PCR (Fig. 1d).

To determine if ER loss following OSM treatment was a permanent effect, we cultured MCF7 and T47D cells with OSM for 48h and then removed cell media, washed cells twice with PBS, and continued culture in cytokine free media for 6, 12, or 24h. As we have noted previously, S100A7 remained elevated in cytokine treated samples at all time points. In contrast, while ER was strongly

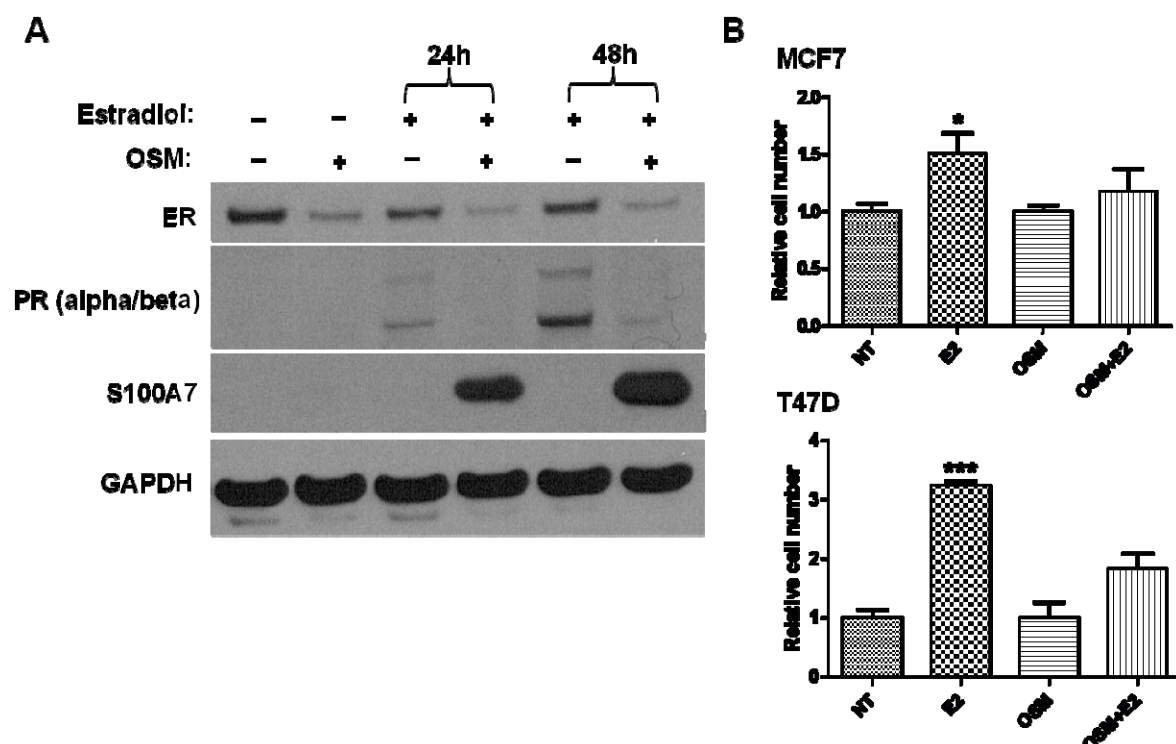
suppressed at 48h of OSM stimulation, its expression largely recovered after 24h in cytokine-free media. This indicates that ER loss is reversible and may not be regulated by the same mechanisms as S100A7.



**Figure 1.** OSM suppresses ER expression and synergizes with TNF. (A) Dose-response western blot experiment in which MCF7 cells were treated at the indicated concentrations of OSM or IL-6 for 48h. (B) Treatment of MCF7 cells with cytokines for 6, 24, or 48h. (C) Treatment of MCF7 cells with cytokines singly or in combination for 24h. (D) Assessment of ER mRNA expression by RT-PCR following OSM treatment for 3 or 24h.

#### *OSM prevents normal cell responses to estradiol treatment*

While OSM consistently suppressed ER expression in our experiments, it rarely did so completely. We therefore wished to know if the observed degree of ER loss was functionally relevant. To address this we performed estradiol stimulation assays in which MCF7 and T47D cells were grown in hormone-free conditions (phenol-red free DMEM with charcoal-stripped FBS) for 3 days, at which point experimental groups were treated with OSM. After a further 24h, cells were treated with 10nM 17-beta-estradiol (E2) for a further 24 or 48h. In the absence of OSM, E2-stimulated cells robustly induced expression of progesterone receptor (PR), a key target gene of estrogen receptor (Fig. 2a). In contrast, OSM strongly prevented induction of PR at both 24 and 48h of E2 stimulation. Furthermore, OSM inhibited the proliferative response of cells to E2, as determined using a fluorescence assay based on Hoechst dye labeling of cell nuclei (Fig. 2b). Intriguingly, under these culture conditions OSM was unable to induce S100A7 expression. However, cells treated with both OSM and E2 robustly expressed S100A7, suggesting that OSM induction of S100A7 is dependent on E2 signaling, possibly through the estrogen receptor beta [8].



**Figure 2.** OSM reduces estrogen response in MCF7 and T47D cells. (A) Expression of PR and S100A7 with respect to estradiol and OSM stimulation (for 24 or 48h) of hormone starved MCF7 cells. (B) Proliferation of MCF7 and T47D cells after 3 days of estradiol (E2) stimulation, with or without OSM. \* $P < 0.05$ , \*\*\* $P < 0.0001$  (Mann-Whitney t-test) relative to untreated control (NT).

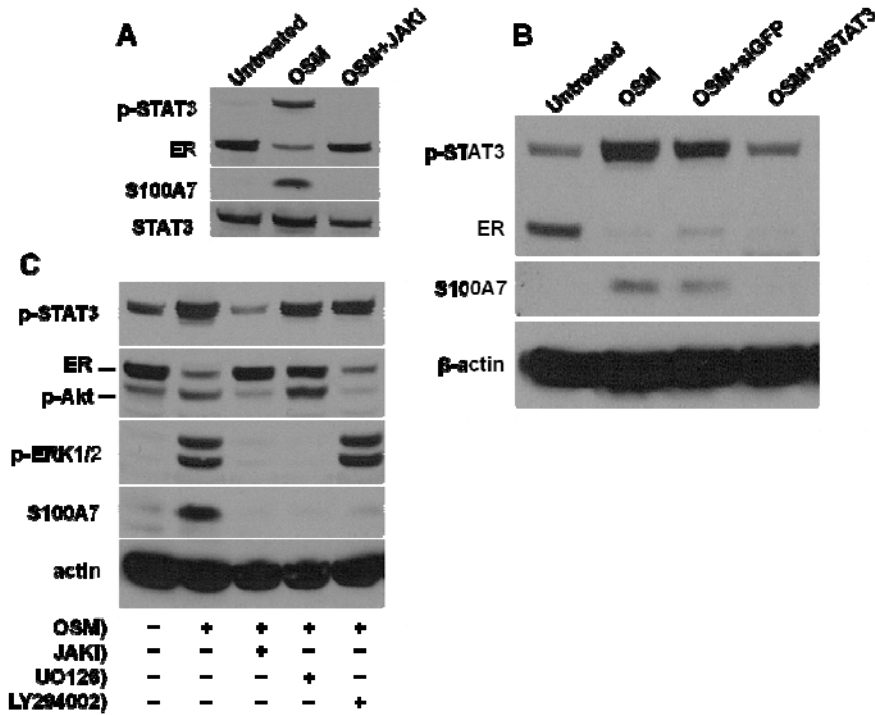
### *OSM suppresses ER expression via the ERK1/2 MAP kinase pathway*

OSM induces signal transduction through a variety of pathways, including JAK-STAT (primarily STAT3), PI3K-Akt-NFkB, ERK1/2 MAPK, and, to some extent, the p38 and JNK MAP kinases [9]. We have previously observed that inhibition of any of the STAT3, PI3K, or ERK1/2 pathways can prevent S100A7 induction by OSM, indicating that all three mechanisms are required in this process [10]. Blockade of JAK signaling in MCF7 cells using a pan-JAK inhibitor completely abrogated the effects of OSM on both S100A7 and ER (Fig. 3a). This was expected, as all signaling from the OSM receptor emanates from JAK activity. In contrast, knockdown of STAT3 expression using siRNA prior to OSM stimulation had no effect on suppression of ER, nor did pharmacological inhibition of the PI3K pathway using LY294002 (Fig. 3b, 3c). Similarly, blockade of the p38 and JNK pathways also had no effect on ER loss. However, blockade of MEKK1/2 (the activating kinases of ERK1/2) using the inhibitor U0126 reproducibly prevented both ER loss and S100A7 induction (Fig. 3c). This is in keeping with prior reports that enforced activation of MAPK signaling can result in ER loss [11-13]. Although NFkB transcriptional activity has been implicated in ER regulation, this was unsupported in our experiments of OSM, as blockade of PI3K (the key upstream kinase of OSM-driven NFkB activity) had no effect on ER. In addition, treatment of cells with oridonin, a specific inhibitor of NFkB DNA-binding, likewise had no influence on ER loss, although it did cause notable suppression of S100A7 induction.

To further rule out other possible pathways of ER suppression we inhibited mTOR activity using rapamycin; mTOR is a key target of both the PI3K pathway (via Akt) and the ERK1/2 pathway. However, rapamycin treatment did not affect ER loss. In addition, we and others [10, 14] have shown a role for signaling from the epidermal growth factor receptor (EGFR) in cooperation with OSMR.



However, inhibition of EGFR signaling using AG1478 again had no effect on ER. Thus, in our hands, OSM causes ER suppression specifically via ERK1/2 signaling, although further details of the mechanism remain unclear.



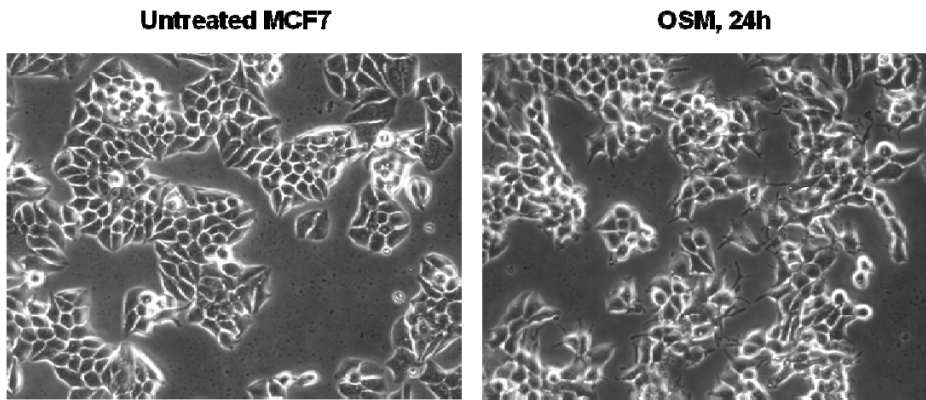
**Figure 3.** OSM inhibits ER expression via the ERK1/2 pathway. (A) Blockade of OSM signaling using a pan-JAK inhibitor. (B) Knockdown of STAT3 with siRNA in the context of OSM stimulation. (C) Blockade of MEKK1/2 and PI3K using U0126 and LY294002, respectively, during OSM stimulation.

#### *OSM does not suppress ER via S100A7*

We have previously reported that S100A7 does not become expressed until approximately 6 hours following OSM stimulation [10]. Because in the current study ER became suppressed at approximately this time point or earlier, it appeared unlikely that S100A7 is responsible for ER loss. To confirm this, we prevented OSM-induced S100A7 expression using S100A7-siRNA and noted no effect on ER expression. Similarly, siRNA knockdown of Jab1, a key functional partner of S100A7, also had no effect on ER suppression. Therefore, although S100A7 is expressed as part of the same program as that which suppresses ER (MAPK signaling), S100A7 is not directly involved in ER regulation.

#### *ER suppression is accompanied by hallmarks of the epithelial to mesenchymal transition (EMT)*

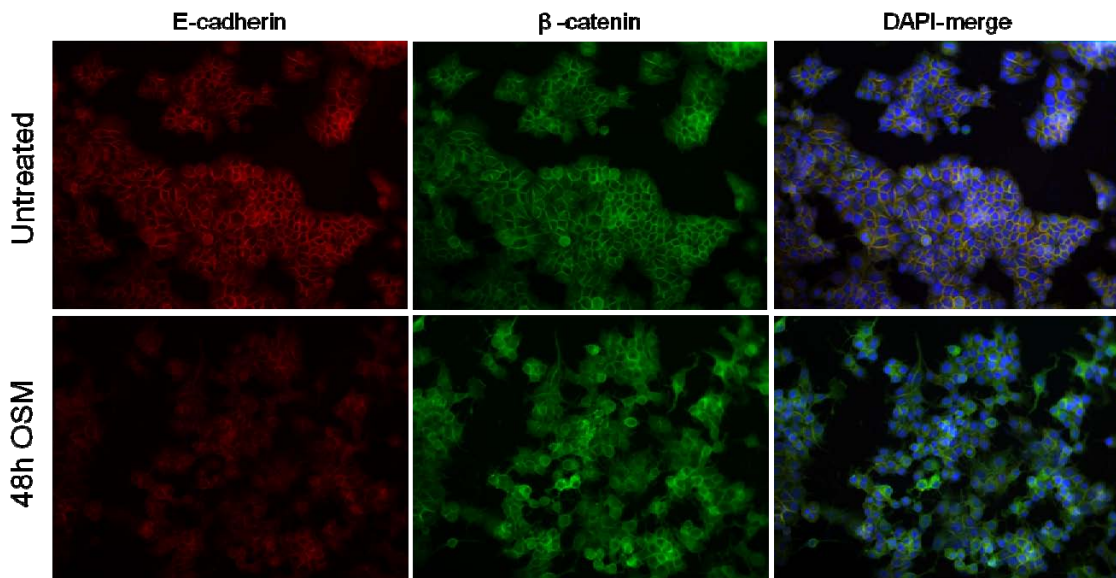
We and others [10, 14-16] have reported that OSM induces a robust phenotypic shift in breast cell lines, including MCF7 and T47D cells. Morphologically, this shift involves a rapid (within 1-2 hours) and marked transition from a typical ‘cobblestone’ epithelial organization to a mesenchymal phenotype, featuring loss of intracellular adhesion and gain of stellate or spicule morphology reminiscent of fibroblasts (Fig. 4). These cells have significantly enhanced motility and invasiveness. Furthermore, OSM has been reported to induce expression of fibronectin (typically considered a mesenchymal product) by MCF7 cells. Together, these observations imply that OSM-treated breast tumor cells undergo an EMT, a process that has gained increasing attention as a putative mechanism for cancer metastasis and treatment resistance. Because this represents loss of epithelial characteristics, the loss of ER (an epithelial differentiation marker) makes intuitive sense in this context.



**Figure 4.** MCF7 cells become mesenchymal in appearance during OSM stimulation.

To further explore the process of EMT in OSM-stimulated breast cells, we examined several additional features. A hallmark of EMT is loss or dysregulation of E-cadherin at cellular tight junctions. We have repeatedly observed in MCF7 cells that total expression of E-cadherin (detected by western blot) is significantly diminished following OSM treatment. Immunofluorescence confirms this and shows that this is particularly evident in cells with mesenchymal morphology.  $\beta$ -catenin is an important intracellular partner of E-cadherin at tight junctions. When E-cadherin is dysregulated,  $\beta$ -catenin becomes liberated and can enter the nucleus to promote mesenchymal gene expression via TCF-LEF transcription factors [17]. While  $\beta$ -catenin shows tight immunofluorescent colocalization with E-cadherin in untreated MCF7 cells,  $\beta$ -catenin becomes much more widely distributed in the cytoplasm and nucleus following OSM treatment (Fig. 5).

The transcription factors snail and twist are thought to be master regulators of EMT. Both of these factors are induced by OSM in MCF7 cells (Fig. 6a). Similarly, although no change is evident in twist expression, snail is robustly expressed by T47D cells following OSM treatment. In sum, the OSM-induced changes described above are highly suggestive of an EMT.

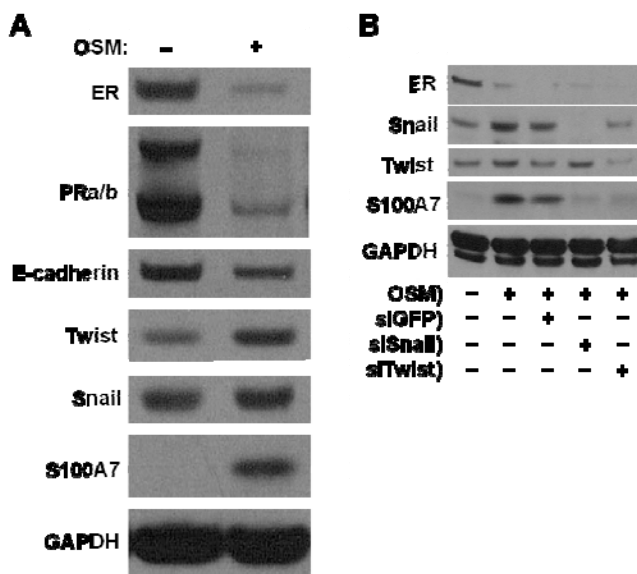


**Figure 5.** OSM disrupts E-cadherin/ $\beta$ -catenin tight junction complexes.

### *Relationships between ER, S100A7, and EMT*

Given that OSM induces S100A7 and suppresses ER in the course of promoting EMT, we wished to know how these features influence one another. We began by blocking induction of snail and twist via siRNA knockdown. Although this intervention did not stop the suppression of ER following OSM treatment, knockdown of both snail and twist prevented induction of S100A7 (Fig. 6b). S100A7 may therefore serve as a marker of cytokine-induced EMT, while ER appears to be suppressed by an alternative mechanism.

To address the role of ER, we have begun experiments involving the overexpression of ER via transfection of an ER-encoding vector (pcDNA3.1). Preliminary experiments indicate that although forced expression of ER does not influence loss of E-cadherin, or induction of S100A7, snail, or twist following OSM treatment, ER-transfected cells nevertheless appear attenuated with respect to acquisition of mesenchymal morphology. This observation is based on gross examination, however. We will shortly be executing migration and invasion assays using this experimental approach to determine if ER loss is indeed a functional component of the EMT phenotype driven by OSM.



**Figure 6.** OSM-induced ER loss occurs in the context of EMT-associated processes. (A) MCF7 cells treated with OSM for 24h lose markers of luminal differentiation (ER, PR, E-cadherin), gain S100A7, and increase expression of the mesenchymal transcription factors snail and twist. (B) Knockdown of snail and twist using siRNA does not prevent OSM-induced ER loss, but does prevent S100A7 induction.

### *Do OSM-stimulated cells acquire stem cell-like characteristics?*

Mounting evidence indicates that both normal and carcinoma cells that have undergone EMT attain features commonly associated with stem cells [18]. These include characteristic patterns of expressed genes, the ability to exclude vital dyes such as Hoechst stain, and enhanced self-renewal capacity under non-differentiating conditions [19]. Based on our observations regarding EMT features induced by OSM, we have recently asked if OSM-treated cells also become more stem-like. Again, the loss of ER in this situation makes intuitive sense, since it would occur in the context of dedifferentiation into a more primitive state. Our preliminary experiment has involved the growth of MCF7 cells as mammospheres, which are spherical colonies of cells grown in suspension in defined media (serum free DMEM, with addition of EGF, bFGF, and insulin). Mammospheres are considered an in vitro measure of stemness, as they are formed by single cells with stem-like characteristics [20]. We have observed that addition of OSM induces rapid formation of mammospheres relative to control conditions. After 10 days of culture,

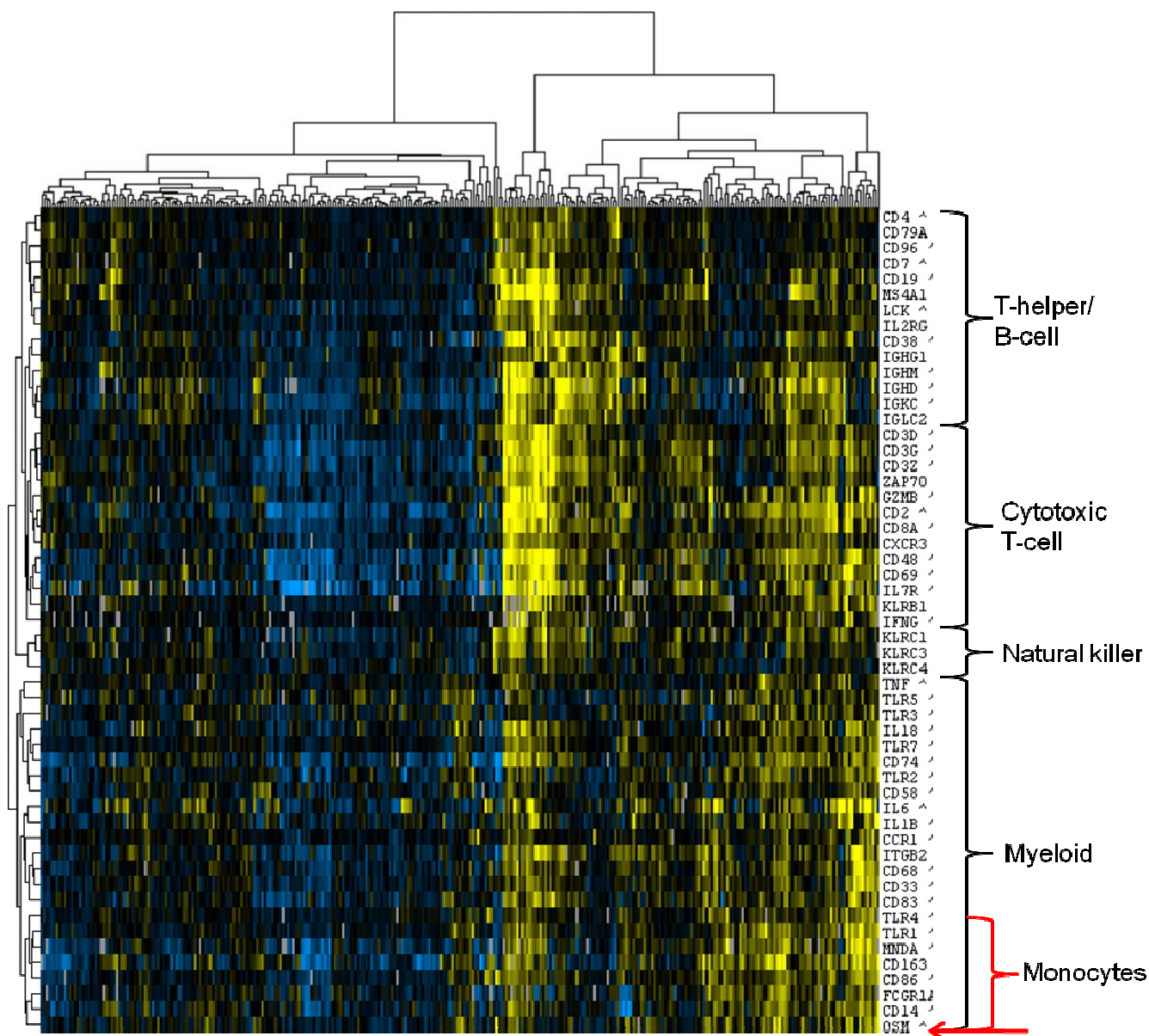
OSM-treated cells expand to populations 2-3 times the size of control cultures. This implies that OSM can enhance the expression of stem cell-like features. We will continue mammosphere assays to verify this, and perform gene expression analyses to assess levels of ER, S100A7, and the EMT-related genes described above, as well as key stem cell transcription factors, including Oct4, Nanog, and Sox2.

#### *Summary of Task B1*

We have demonstrated that OSM is a potent regulator of ER expression in vitro, controlling ER expression specifically through the ERK1/2 MAPK cascade. This relates to our previous study demonstrating that OSM induces S100A7, a tumor promoter gene associated with ER negativity in vivo. ER loss appears to be reversible upon removal of cytokine stimulation, indicating that cells retain phenotypic plasticity. Loss of ER and gain of S100A7 appears to take place in conjunction with the broader phenomenon of OSM-induced EMT. S100A7, in fact, appears to be controlled by master EMT transcription factors. In sum, loss of ER and gain of S100A7 in response to OSM may be directly related to the process of dedifferentiation into a mesenchymal state.

#### **Task B2**

Clinical case cohorts for assessment of OSM and OSMR expression in tissues are under construction, but not yet completed. However, we have performed in silico analyses of publically available gene expression data to generate observations in the interim. We have primarily utilized a previously published breast cancer dataset (n~320) analyzed by gene expression microarray on the Agilent platform [21]. These data were downloaded from the University of North Carolina microarray database. The primary aim of Task B2 was to identify the origin of OSM in breast cancer tissues. As previous reports have indicated that OSM can be produced by activated monocytes (myeloid lineage) and helper T cells (lymphoid lineage), we asked whether OSM expression in these samples was correlated with specific subtypes of leukocytes. We compiled a list of approximately 50 genes (median normalized) that are known to be expressed by distinct leukocyte subsets and, along with OSM, used them for unsupervised hierarchical clustering using uncentered correlation and complete linkage (Fig. 7). By this approach, several logical gene clusters emerged, including one associated with T-helper and B-cells (genes including *CD4*, *CD79A*, *MS4A1*, *CD19*, and immunoglobulins), one associated with cytotoxic T-cells (genes including *CD8A*, *CD2*, *GZMB*, and *IFNG*), one associated with natural killer cells (killer cell lectin genes), and one large cluster associated with genes of the myeloid lineage (*CD68*, *CD14*, *CD163*, *TLR4*, etc). Thus, our approach resulted in an expected pattern of gene clusters. *OSM* clustered discretely with genes of the myeloid lineage, doing so most strongly with the following: *CD14*, *CD86*, *CD163*, *MNDA*, *FCGR1A*, *TLR1*, and *TLR4*. This implies that, in this particular cohort, OSM is associated primarily with myeloid cells, most likely monocytes/macrophages. Nevertheless, this does not preclude the possibility that OSM could be produced by other cells types in breast tumors, including by the malignant cells themselves. This will be made clearer by our analysis of the clinical cohorts currently under construction.



**Figure 7.** OSM expression correlates with genes associated with monocytes. Genes (y-axis, median-normalized) were clustered in an unsupervised fashion using uncentered correlation and complete linkage. Yellow indicates over-expressed genes; blue underexpressed. Gene clusters are labelled based on putative cell(s) of origin. The red arrow indicates OSM.

### Task B3

As indicated above, construction of in-house cohorts for clinical analysis are underway. In the interim, we have pursued clinical questions using the microarray cohort described above. We first wished to verify that the *OSM* pathway was associated with ER loss in vivo. We began by categorizing cases based on high and low (cut at median) expression of *OSM* and *OSMR*, as follows: low expression of both *OSM* and *OSMR* (lo/lo), high expression of *OSM* or *OSMR* (hi/lo), and high expression of both *OSM* and *OSMR* (hi/hi). In support of our in vitro data, there was a dramatic trend towards decreased expression of the ER-alpha gene (*ESR1*) in hi/lo and hi/hi cases (Mann-Whitney T-test; both  $P < 0.0001$ ). Similarly, genes regulated by ER were also reduced in hi/lo and hi/hi cases, including *PGR* (PR), *CCND1*, and *TFF1*, indicating that the estrogen responsiveness of these cases was disrupted (Fig. 8a). With regard to molecular subgroups, Hi/lo and Hi/Hi cases had a propensity towards being categorized as either basal-

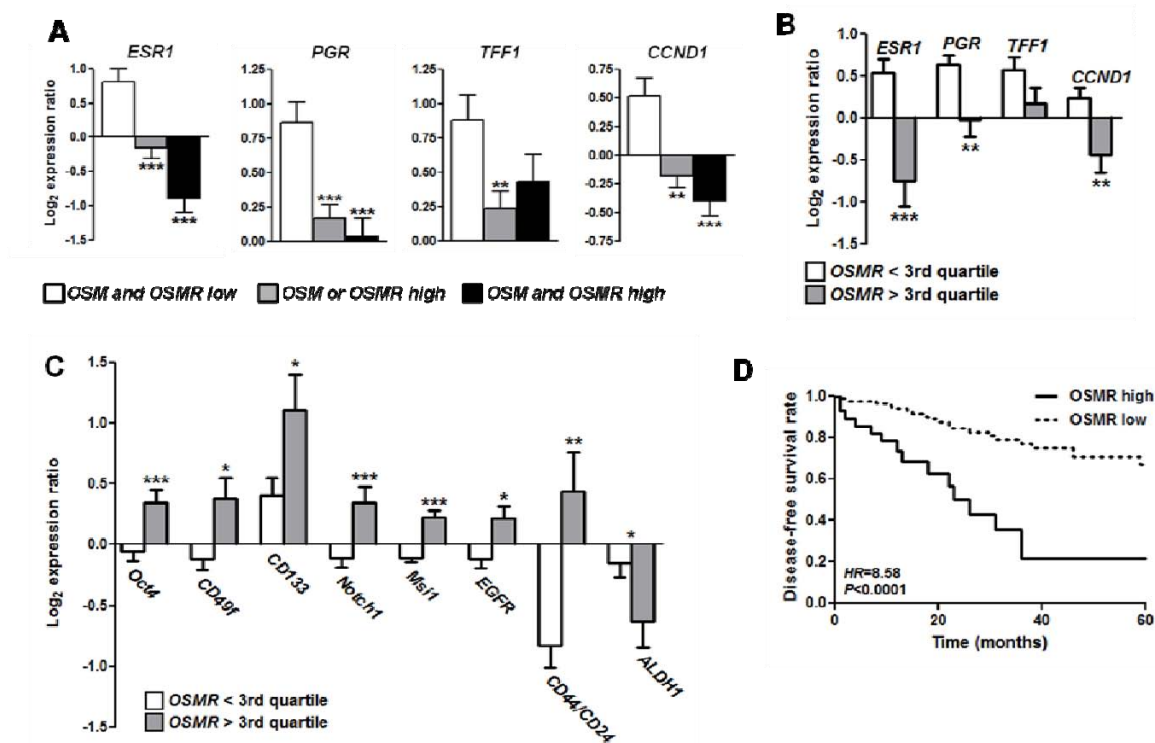
like, claudin-low (a category similar to basal-like but with enhanced expression of lymphocyte and stem-cell genes), or Her2+ ( $\chi^2$  test;  $P<0.0001$ ), all of which are subtypes associated with loss of ER expression and poor prognosis. The OSM axis was also strongly associated with the clinically important triple negative phenotype (lack of clinically assessed ER, PR, and Her2); indeed, the vast majority of triple negative cases (91%) had high expression of either *OSM* or *OSMR* (Fisher's exact test;  $P<0.0001$ ).

A caveat of the above analyses is that, as we have already shown, OSM correlates strongly with myeloid cells. Furthermore, OSMR can be inducibly expressed by macrophages [22]. Therefore, we re-assessed the correlation of the OSM axis with ER genes in cases with less than the median expression level of *CD68* (a key macrophage marker), in an attempt to enrich for cases that expressed OSMR predominantly in the epithelial compartment. As in the entire cohort, the OSM axis maintained a strong association with loss of ER sensitivity.

To determine the relative importance of *OSM* versus *OSMR*, we assessed these genes individually in the *CD68*-low subgroup (the upper quartile was used to define high-low status). Intriguingly, only *OSMR* exhibited consistent association with loss of ER and ER-regulated genes, suggesting that, provided OSMR is expressed at high levels, breast tumor cells can respond to varying concentrations of OSM, rendering the actual level of OSM itself less important (Fig. 8b). We also noted that cases with high *OSMR* had strong expression of genes associated with breast stem or progenitor cells (Fig. 8c), including *POU5F1* (Oct4), *ITGA6* (CD49f), *PROM1* (CD133), *NOTCH1*, and *MSI1*, supporting the idea that OSM signaling can induce a stem-like state in breast tumor cells, one that was developed from our in vitro observations. In keeping with the observations described thus far, *OSMR*-high patients had strongly reduced 5-year disease free survival in both the total cohort (log rank HR=3.24;  $P=0.0001$ ) and in the *CD68*-low subset (log rank HR=8.58,  $P<0.0001$ ; Fig. 8d). In multivariate Cox-regression analysis involving standard clinical parameters, *OSMR* maintained strong independent prognostic significance ( $P=0.009$ ). The OSM pathway is therefore associated with aggressive features both in vitro and in vivo, ultimately contributing to poor patient survival.

To determine the importance of OSMR expression as compared to the receptors of other key inflammatory cytokines, we examined expression of the IL-6 receptor alpha chain (*IL6R*), the LIF receptor alpha chain (*LIFR*, which can also serve as an alternative receptor for OSM), the IL-1 receptor (*IL1R1*), and the TNF receptor (*TNFRSF1B*). These were again examined in the *CD68*-low group, due to their strong correlations with myeloid cells. Unlike *OSMR*, none of these four receptors exhibited consistent associations with ER-associated genes, nor with stem cell-related genes. These receptors likewise failed to correlate significantly with patient survival. The OSM pathway may therefore be a uniquely vital pathway of inflammation-induced tumor progression in breast cancer.

Although data from in-house patient cohorts with respect to the OSM pathway are pending, the data outlined above will serve as valuable supporting information for one or more publications with anticipated submission within the next 12 months.



**Figure 8.** The OSM pathway correlates with ER-negativity and poor prognosis in vivo. **(A)** Association of combined OSM and OSMR expression status with ER-related genes in the total cohort. **(B)** Association of OSMR with ER-related genes in the CD68-low subset. **(C)** Association of OSMR with stem/progenitor cell-related genes in the CD68-low subset. **(D)** Kaplan-Meier curve of disease-free survival in CD68-low patients according to OSMR expression status. \* $P<0.05$ , \*\* $P<0.01$  to 0.001, \*\*\* $P<0.001$  (Mann-Whitney t-test), relative to OSM/OSMR low groups.

#### Task B4.

As in tasks B2 and B3, cohorts are currently under construction, but no data is available as of yet.



## Key research accomplishments over the period Jan-2010 to Dec-2010

- Publication of results associated with Task A3: West, NR and Watson, PH. 2010. S100A7 (psoriasin) is induced by the proinflammatory cytokines oncostatin-M and interleukin-6 in human breast cancer. *Oncogene* **29**; 2083-92.
- Attendance of the AACR Translational Cancer Medicine Conference—March 21–24, 2010; Amsterdam, the Netherlands. Awarded an Aflac-AACR Scholar-in-Training Award (2010) for presentation of the following abstract: West NR, Milne K, Truong PT, Nelson BH, Watson PH. TIA1<sup>+</sup> and FOXP3<sup>+</sup> tumor infiltrating lymphocytes are positive prognostic factors in estrogen receptor negative breast cancer. *See last bulleted point in this section (subsection (i); note that the manuscript title has changed since presentation of this abstract).*
- Presentation of the above abstract at the ImmunoVancouver Meeting 2010, June 28, 2010; Vancouver, BC, Canada.
- Work supported by this award formed the basis of a successful project grant proposal: *Role of the OSM-S100A7 pathway in breast cancer progression*. Canadian Breast Cancer Foundation; \$166,676 over two years.
- Established that OSM exerts a potent de-differentiation effect on breast cancer cells, resulting in loss of hormone receptors (with emphasis on ER) and gain of S100A7 and mesenchymal features. This process may occur as part of an epithelial-to-mesenchymal transition with possible reversion into a stem cell-like state. Data expected to be submitted for publication within this quarter.
- In a large clinical cohort, revealed a robust association between the OSM pathway and the following features: (i) loss of expression of ER and ER-regulated genes; (ii) increased prominence in clinical and molecular subtypes known for heightened aggressiveness (particularly the triple negative and basal/claudin-low subtypes); (iii) expression of genes consistent with a de-differentiated, stem cell-like state; (iv) poor prognosis.
- First author status on two clinical manuscripts at the stage of submission or under review:
  - i) Nathan R. West, Katy Milne, Pauline T. Truong, Nicol Macpherson, Brad H. Nelson, & Peter H. Watson. Tumor infiltrating lymphocytes predict response to anthracycline-based chemotherapy in estrogen receptor-negative breast cancer. In preparation for submission to *Breast Cancer Research*.
  - ii) Nathan R West, Valerie Panet-Raymond, Pauline T Truong, Cheryl Alexander, Sindy Babinszky, Katy Milne, Louetta A Ross, Steven Loken, & Peter H Watson. Intratumoral immune responses can distinguish new primary and true recurrence types of ipsilateral breast tumor recurrences (IBTR). Under review with *The Breast*.



## Reportable outcomes over the period Jan-2010 to Dec-2010

- West, NR and Watson, PH. 2010. S100A7 (psoriasin) is induced by the proinflammatory cytokines oncostatin-M and interleukin-6 in human breast cancer. *Oncogene* **29**; 2083-92.
- West NR, Milne K, Truong PT, Nelson BH, Watson PH. TIA1<sup>+</sup> and FOXP3<sup>+</sup> tumor infiltrating lymphocytes are positive prognostic factors in estrogen receptor negative breast cancer. AACR Translational Cancer Medicine Conference—March 21–24, 2010; Amsterdam, the Netherlands.
- West NR, Milne K, Truong PT, Nelson BH, Watson PH. TIA1<sup>+</sup> and FOXP3<sup>+</sup> tumor infiltrating lymphocytes are positive prognostic factors in estrogen receptor negative breast cancer. ImmunoVancouver Meeting 2010, June 28, 2010; Vancouver, BC, Canada.
- Accrual of operating grant funding: *Role of the OSM-S100A7 pathway in breast cancer progression*. Canadian Breast Cancer Foundation; \$166,676 over two years.

## Conclusions

Tasks A1 and A3 were completed during the first reporting period. Tasks A2/A4/A5 have been delayed pending production of reliable human S100A7. Having generated a successful HEK-based protein expression system, however, efforts to produce fully human-derived S100A7 are now underway. Execution of Task B1 has resulted in a considerable dataset demonstrating that OSM induces simultaneous upregulation of S100A7 and downregulation of ER through an ERK1/2-dependent mechanism. This appears to occur in the context of EMT, with possible acquisition of stem cell-like features. Tasks B2/B3/B4 are underway, but no data are currently available from in-house patient cohorts. However, preliminary work has been performed with respect to tasks B2 and B3 using publically available data from breast cancer transcriptional profiling. This has revealed that OSM may be produced primarily by cells of the monocyte lineage in breast cancer tissues (Task B2). In addition, the OSM pathway appears to be strongly associated with loss of ER (and the molecular subtypes associated with this) and poor patient prognosis.

Our work has begun to reveal that OSM can induce multiple features consistent with aggressiveness in breast cancer, including loss of ER and gain of S100A7. From a broad biological perspective, this begins to shed light on the etiology of breast cancer heterogeneity. The concept that distinct breast cancer subsets arise from distinct progenitor cells with unique patterns of differentiation has become popular in recent years [4, 19]. However, our data suggest that breast cancer cells may have a remarkable degree of phenotypic plasticity, and are strongly influenced by environmental factors. This is demonstrated by our observation that breast tumor cells lose ER and estrogen responsiveness following relatively brief exposure to OSM, but can rapidly reverse this process following withdrawal of stimulation. This information challenges the common assumption that tumors progress strictly via accumulation of mutations. In addition, because OSM may be a relatively redundant mechanism during the inflammatory cascade, targeting of this pathway in breast cancer could provide a favorable therapeutic approach (via maintenance of hormone responsiveness and suppression of metastasis) with minimal adverse effects.

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